(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 986 960 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
22.03.2000 Bulletin 2000/12

(51) Int Cl.7: **A23J 1/00**, A23J 3/22, A23J 3/20

(21) Application number: 98307450.1

(22) Date of filing: 15.09.1998

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

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(54) Mucorales fungi for use in preparation of textured products for foodstuffs

(57) The preparation of a proteinaceous substance suitable for use in a foodstuff is described which comprises fungal fells of the order *Mucorales*. The cells are grown in a fermentor vessel in a liquid which is mixed during fermentation, after which the RNA content of the fungal cells is reduced and the fungal cells processed into an edible substance. This substance is then mechanically texturized into edible textured product for inclusion into foodstuffs, for example in the form of chunks as a meat substitute.

Printed by Jouve, 75001 PARIS (FR)

Description

Field of the Invention

[0001] The present invention relates to the preparation of edible proteinaceous substances, for use in making textured products, using fungal cells of the order *Mucorales* and the use of these substances and products in foodstuffs in particular as meat substitutes.

Introduction

[0002] Animal meat is considered to be a desirable part of the human diet, not only due to the vitamins and nutrients it provides, but also due to its flavour (particularly on cooking) and, importantly, its texture. However, an increasing number of people are turning to vegetarian or vegan diets, neither of which can include meat or meat derived products. Such diets may be due to a number of factors, but is often due to either a disliking for meat (either in texture or flavour) or due to ethical and moral considerations (for example, a belief that it is wrong to kill animals in order to feed humans).

[0003] The move towards vegetarian/vegan diets has increased in recent years by the appearance of BSE (Bovine Spongiform Encephalopathy), otherwise known as "mad cow disease", a disease that effects the nervous system, in particular the brain, that has appeared in cows and is thought to be as a result of feeding cattle parts of sheep infected with a similar disease known as "scrapie".

[0004] A number of edible meat substitutes or meat replacements have been proposed in recent years. Soy-based products, in particular extruded soy, are marketed, especially by American and Japanese companies, but these do not have a particularly meat-like texture or taste.

[0005] EP-A-0,123,434 (Ranks Hovis McDougall) proposed the production of edible protein-containing substances by the production of mycelial protein by the fungus *Fusarium graminearum*. These substances have been increasingly used as meat substitutes, and are included in foodstuffs sold under the trade mark Quorn™.

[0006] Various ways of producing proteinaceous foods by fungal fermentation are already known. One example is the traditional Indonesian fermented food, tempeh. This is usually prepared by the fermentation of *Rhizopus* fungi on soy beans and parts thereof acting as a moist solid substrate. The beans (or other vegetable substrate) are inoculated with the fungus and fermentation allowed for 24 to 36 hours. The beans become bound by the fungal mycelium protein produced to give a firm product which can then be sliced before eating (no additional processing is usually performed before consumption). Thus the fungi are used to hydrolyse an otherwise inedible substrate and, apart from thus lacking much taste or flavour, tempeh is relatively dry and does not have the fibrous and juicy texture associated with meat. It is thus not particularly appealing as a meat substitute, at least for Westerners.

[0007] GB-A-2007077 (Maclennan/BioEnterprises) proposes a similar process to the manufacture of tempeh, except instead of soy beans the solid substrate is a starch-containing food such as sago, cereals or potatoes. However a prerequisite of this foodstuff (and tempeh) is that solid foods or ingredients are needed as the substrate for the fermenting microorganisms.

Brief Description of the Invention

[0008] According to a first aspect of the invention there is provided a process for the preparation of a proteinaceous substance, suitable for use in a foodstuff, comprising fungal cells, the process comprising:

a. fermenting fungal cells of the order *Mucorales* in an aqueous liquid contained in a fermenter vessel, the liquid comprising an assimilable nitrogen (N) source and an assimilable carbon (C) source which allows the fungal cells to produce protein, and mixing (and optionally aerating) the liquid and cells during fermentation;
b. reducing the RNA content of the fungal cells;
c. before or after (b),, removing at least some of the water from the fungal cells; and
d. processing the fungal cells into an edible substance.

[0009] By using (non-toxic) *Mucorales* fungi (used in Asian fermented food products) one can avoid any mycotoxins that may be produced by other (e.g. prior art) organisms. Thus little or no screening for organisms that are safe for inclusion into foodstuffs may be required. This means that the products produced by the invention are more suitable for ingestion and for use in foodstuffs. The *Mucorales* organisms in general do not produce mycotoxins, which is clearly advantageous as these organisms are incorporated whole into a foodstuff, and can mean that the processing techniques can be more efficient as mycotoxins may not need to be removed.

[0010] A further advantage of using *Mucorales* fungi is that a relatively wide variety of microorganisms is available, depending upon the characteristics desired in the proteinaceous substance. This can allow differing physical charac-

teristics (such as in the fibrous nature, or mouthfeel) or in chemical characteristics (taste etc). The fungi used in the present invention have been found to give improved meat-like properties, for example a more fibrous juicy texture. Therefore by choice of microorganism one can produce a proteinaceous substance that has desirable characteristics according on the eventual foodstuff to be prepared. In particular, different microorganisms can have different colours, and so as well as being able to prepare a white substance, one can make substances that have a yellow or green appearance, which may be desirable for some foodstuffs.

[0011] Mycotoxins anticipated here are those such as aflatoxin, mevinolin and terrein. A fungus that produces any of these mycotoxins is unlikely to be allowed to be used in any form of food production, even if the manufacturer takes steps to remove the mycotoxins. It is therefore particularly important to choose fungi that will not produce these mycotoxins at any stage of the process.

[0012] The fungi can be of the family *Choanephoraceae,* such as of the genus *Blakeslea,* for example of the species *Blakeslea trispora.* Other three families included within the order *Mucorales* are *Cunninghamellaceae, Mortierellaceae* (such as fungi of the genus *Mortierella,* and in particular the species *Mortierella alpina)* and, especially, *Mucoraceae.*

[0013] Preferred fungi are saprophytic (that is to say, simple fungi) rather than parasitic (which are more complex). The "simple" fungi are usually preferred because they are better adapted towards hyphal growth, whereas the parasitic organisms concentrate on taking nutrients from their "host" organism.

[0014] The fungal cells are preferably of the genus *Rhizopus, Rhizomucor, Mucor* or *Mortierella,* all of which belong to the family *Mucoraceae.* Suitable fungi of the genus *Rhizopus* include *Rhizopus stolonifer, Rhizopus arrbizus, Rhizopus miehei, Rhizopus pusillus, Rhizopus oligosporus* and, in particular, *Rhizopus oryzae.*

[0015] Of the *Rhizomucor* genus, a preferred fungi is *Rhizomucor miehei:* some *Rhizomucor* (e.g. *pusillus*) strains may be excluded, for example if they are thought to be thermophilic (for instance they may grow at 40°C). Thermophilic organisms are, therefore, generally not to be used in the present invention.

[0016] Preferred fungi can have a cell wall comprising, or primarily containing, chitin and chitosan. The cell walls may contain one or more of the sugars glucosamine (such as D-glucosamine) and/or fucose, such as L-fucose, and may be substantially free of galactose.

[0017] The fungi used in the present invention preferably do not have septa, which is in contrast to those used in Quorn™ (of the group *Fusarium*). Furthermore, preferred fungi have branching, again unlike *Fusarium* organisms which have little or no branching (in their hyphae). Indeed, the art advocates the use of non-branching mutants (EP-A-0,123,434). In the present invention the fungi suitably produce hyphae, usually with lengths of from 0.1 to 6mm, such as from 0.2 to 5mm. The hyphae may have a diameter of from 1 to 20 μm, such as from 2 to 10 μm, optimally from 2 to 8 μm.

[0018] The fungus is preferably a filamentous fungus (or mould), suitably capable of producing microfungal fibres (or mycelia). These are usually edible (and digestible) by humans or animals.

[0019] The fungus may be a naturally occurring one, it may have been selected using known techniques for particular desired properties, or it may be genetically engineered.

[0020] Usually fungi of the class *perfecti* (in other words, not belonging to the class *imperfecti*) will be employed, which are able to reproduce sexually. For this reason *Rhizopus oryzae* and *Rhizomucor miehei* are preferred. One or more fungi of the order *Mucorales* may be used in the invention.

[0021] As will be appreciated, the process of the first aspect is a liquid fermentation, in other words the aqueous liquid serves as-the fermentation medium, for example an aqueous solution. This contrasts with prior art processes which culture fungi on a solid substrate, that substrate being, for example, rice, soy bean or starch-containing products such as cereals or potatoes. In the invention the liquid fermentation process in (a) is conducted preferably in the absence of a substrate which is itself an edible foodstuff (which includes not only vegetable material but also meat) or is a solid.

[0022] The fungal cells will preferably remain intact (or whole) not only during the fermentation process, but also during subsequent processing steps, including water removal, reduction of RNA content and any texturing. Thus the substance (or textured product) will contain intact (but dead) fungal cells, and during most if not all stages of the preparation of the substance or the product, steps will be taken to minimize damage to and lysis of the cells or cell membranes. However, during processing some compounds may leave the cell (so that the cell membrane may "leak" a little). It is of course intended that the process of the invention involves the removal of some RNA, and preferably also water, from the fungal cells.

[0023] A second aspect of the present invention relates to the preparation of an edible textured (proteinaceous) product, the process comprising mechanically texturising an edible proteinaceous substance comprising fungal cells of the order *Mucorales* having a reduced RNA content.

[0024] The substance (and so also the textured product) may also contain fungal protein produced by the fungal cells. This protein may be intracellular, within the cell membrane and/or extracellular. Here, the proteinaceous substance, for example a biomass, can be one that is preparable by the process of the first aspect.

[0025] A third aspect of the present invention relates to a proteinaceous substance, suitable for use in a foodstuff (thus it can be edible, and also suitably digestible) comprising fungal cells of the order *Mucorales* having a reduced

RNA content. The substance can be devoid of solid starch-containing material or vegetable or vegetable derived material or indeed any edible material (other than the fungal cells and protein).

[0026] The fungal cells preferably constitute 40%, e.g. at least 50% or even 60% or more of the proteinaceous substance. However these amounts can be much larger and the cells can constitute at least 70%, such as at least 80%, and optimally at least 90% of the proteinaceous substance. With such a high content of fungal cells one can obtain a substance (and, also a textured product) that is more juicy, fibrous and better tasting. Even after texturing the resulting edible textured product can have a fungal cell content the same as that quoted for the proteinaceous substance (except the percentages are then based on dry weight of the cells in the product).

[0027] The substance can therefore consist essentially only of the fungal cells (which can include the protein produced by those cells). During fermentation, therefore, there will usually be no extraction of isolation of any particular compound(s) or substance(s) either contained in or produced by the fungi as the fungal cells. Indeed in the processes of the invention it is preferable that RNA (or any degradation products thereof or any compounds undesirable during processing) will be the only compound(s) that is removed from the cells.

[0028] The proteinaceous substance of the third aspect is edible in the sense that it can be included into a foodstuff or is compatible with food use. Although it can be therefore eaten as such, the intention is that this proteinaceous substance is in fact an intermediate in the preparation of a textured product which forms the fourth aspect. This aspect thus encompasses an edible textured product comprising fungal cells of the class *Mucorales* having a reduced RNA content. This product can be preparable by the process of the second aspect.

[0029] The proteinaceous substance may have, as the only edible material, the fungal cells and so may, apart from the fungal cells, be devoid of edible substances. However, various edible component(s) can then be mixed with or added to the proteinaceous substance of the third aspect to produce the textured product of the fourth aspect. Further or additional edible component(s) may be added to the textured product in the preparation of the foodstuff of the fifth aspect.

Detailed Description of the Invention

[0030] The fermentation process of the first aspect is suitably conducted in a fermenter vessel adapted for containing the aqueous liquid, such as a vat, and this vessel may be pressurized. It may be also be adapted to allow-the continuous or continual supply of the assimilable nitrogen and/or carbon sources. Stage (a) and later stages are therefore preferably conducted aseptically. Although the fermentation can be a continuous process, with regular harvesting or removal of the fungal cells (and accompanying protein), the process can be a batch process if desired. Thus the fermentation process can be stopped or halted, and the fungal cells removed from the vessel, before another or fresh fermentation is begun.

[0031] The vessel may additionally be adapted to perform, or allow to be conducted, aeration and/or mixing of the cells and liquid, such as agitation of the solution, which may be stirring, for example achieved using mechanical means.

[0032] The carbon and nitrogen sources may be provided in separate compositions. This because the different sources may be subject to different sterilizing conditions, and furthermore it allows a variation in the relative amounts of carbon and nitrogen during fermentation.

[0033] The nitrogen and/or carbon sources can be supplied (or added) separately, or supplied simultaneously, or supplied as a combined preparation. They may thus present in the same composition (if thought necessary) which is preferably a liquid. The C and/or N sources can be added (to the fermenter vessel) either before the fungal cells are added (to the vessel), in other words prior to inoculation, or during fermentation.

[0034] If the supply is continual (or intermittent), it is preferred that for each instance of supply (e.g. "shots" or additions) the addition of both carbon and/or nitrogen sources is the same.

[0035] Preferred C:N (weight) ratios are at least 6:1, but may vary from 10:1 to 150:1, such as from 15:1 to 50:1, optimally from 25:1 to 40:1.

[0036] For continual supply, preferably the time during which the nitrogen and/or carbon sources are supplied are greater than the time when they are not. Thus, during fermentation supply is advantageous for at least 50% of the time. If supply of one or both sources is intermittent, then there should be at least 2, preferably at least 5, and optimally at least 10, additions to the aqueous liquid of the nitrogen and/or carbon source.

[0037] The carbon and/or nitrogen sources may be complex sources, or individual or isolated compounds. Non-complex sources are preferred (these may have or produce fewer mycotoxins) and so in the latter two cases these may be added in a high degree of purity, and can be common (or commercially available) chemicals. Preferably both C and/or N sources are not solid, and suitably both are liquids.

[0038] Suitable nitrogen sources include ammonia or ammonium ions. The advantage here is that ammonia can act as a pH regulant. This may be supplied in the form of an ammonium salt, such as nitrate, sulphate or phosphate or in the form of ammonium ions themselves, for example an aqueous solution of ammonium hydroxide.

[0039] Other inorganic nitrogen sources can also be used, such as sodium nitrate, urea or an amino acid such as

asparagine or glutamine.

[0040] Other complex sources include yeast hydrolysates, primary yeast, soy bean meal, hydrolysates of casein, yeast, yeast extract or rice bran.

[0041] The carbon source can comprise (complex sources such as) maltodextrin, oat flour, oat meal, molasses, vegetable (e.g. soy bean) oil, malt extract, starch, ethanol or soy bean oil. Preferred (non-complex) carbon sources include carbohydrates or sugars, such as fructose, maltose, sucrose, xylose, mannitol, glucose or lactose or glycerine (e.g. from a vegetable source), citrate, acetate, glycerol, ethanol or (e.g. sodium) ascorbate.

[0042] Preferred nitrogen and/or carbon sources are water soluble or water miscible.

[0043] The aqueous liquid may additionally contain other substances to assist in the fermentation, for example a chelating agent (e.g. citric acid), an anti-foaming agent (e.g. soy bean oil), a vitamin (e.g. thiamine and/or riboflavin), any necessary catalytic metals (for example, alkali earth metals such as magnesium or calcium, or zinc or iron and/or other metals such as cobalt and copper), phosphorus (e.g. phosphate) and/or sulphur (e.g. sulphate). Preferably the aqueous liquid will have a low sulphur content, for example less than 3.0g, preferably less than 1.0g, of sulphur by litre of aqueous liquid. Optimally the level is below 0.4g sulphur per litre of aqueous liquid.

[0044] Preferably, the pH, temperature and/or oxygen content (of the aqueous liquid) during fermentation is controlled. This may be to keep the pH, temperature and/or O_2 content constant or within a desired range. In this respect, the fermented vessel may have pH, temperature and/or O_2 content sensors.

[0045] The pH of the aqueous liquid during fermentation may be from 4 to 10, such as from 5 to 8, optimally from 6 to 7.

[0046] The temperature of the aqueous liquid during fermentation is not particularly critical, but may be from 20 to 40°C, such as from 25 to 37°C, optimally from 30 to 35°C.

[0047] It can be important that during fermentation mixing occurs. In other words, the aqueous liquid and fungal cells are suitably either mixed or agitated. This may be achieved if aeration is provided, in other words by bubbling air into the aqueous liquid. This may serve the additional purpose of providing oxygen to the fungal cells: hence the fermentation is preferably an aerobic one.

[0048] Other means of agitation or mixing include stirring, for example using an impeller. This may be of a hydrofoil axial flow design or may be designed so that the aqueous medium is forced radially outwards from the impeller (such as a turbine). Even if there is no stirring it is preferred that the fungi are provided with oxygen during fermentation, and so aeration (e.g. by bubbling air, O_2 or other oxygen-containing gas) is advantageous here. Aeration may be at from 0.1 to 2.0, such as from 0.5 to 1.0 vvm.

[0049] One of the advantages of aeration and/or agitation is that the oxygen content of the aqueous liquid can be kept relatively high. This may be at least 10%, such as at least 15%, optimally at least 20% (in terms of air saturation). This allows a more efficient formation process, and can thus result in a quicker and/or higher content of fungal cells and/or fungal protein. This is particularly advantageous for fungal cells used in the invention because these are sufficiently robust to allow agitation and/or mixing during fermentation. This is not always possible however with (the more sensitive) fungal cells of the group *Fusarium*. Thus with *Mucorales* organisms one does not have to use expensive equipment, such as airlift fermenters, developed for other (less robust) organisms, which means the edible substance can be produced more cheaply.

[0050] The fermentation may take from 1 to 12 days, such as from 2 to 6 days, and optimally from 2 to 4 days. A shorter fermentation lends itself towards a batch, rather than continuous, fermentation process.

[0051] Once fermentation has finished, or fermentation is to be stopped, water can be removed from the combination of the fungal cells and the surrounding liquid produced. In the art this combination of aqueous liquid and fungal cells is often referred to as a "broth". During fermentation the vessel should contain only this broth, and this is preferably entirely liquid (and so devoid of any solid material). The cells may be rinsed, such as with an aqueous liquid e.g. water, before or after this water removal stage, and either or both may result in the separation of the cells from extracellular matter (e.g.protein) if desired. If necessary depelleting (the dispersion or minimisation of any pellets in the liquid) may be conducted before water removal (For example by sonication or shear mixing).

[0052] Water removal is preferably by mechanical means or by mechanical techniques. These include various solid-liquid separation techniques such as mechanical de-watering, filtration, centrifugation (preferred), settling (in other words, the material is allowed to settle, thus using gravity), heating or drying.

[0053] After this de-watering the water content can be from 50 to 90%, such as 60 to 85%, optimally from 70 to 80%.

[0054] Following this (optional) de-watering, the RNA content of the fungal cells can then be reduced. This can be achieved by chemical and/or physical methods. The preferred method is to use and so take advantage of one or more enzymes already inside the fungal cells to digest the RNA. This may allow any resulting (small) RNA molecules (or degradation products thereof) to pass through, and so outside, the cell membrane. Suitably the (undesirable) nucleotides inside the cell are cleaved into 2, 3 and 5-nucleotides; thus it may be these nucleotides that are transported through the cell membrane. Dewatering may also remove other compounds not desired during further processing, such as glucose (for example due to later heat treatment).

[0055] A preferred method of RNA removal is heat treatment, in other words heating the fungal cells. This may have

two effects. Firstly, the cell becomes more permeable, allowing RNA and other molecules to pass outside the cell. It may also increase the activity of nucleases, such as RNAases, inside the fungal cells. A further advantage is that such heat treatment may inactivate any undesirable enzymes inside the fungal cells. Alternatively or in addition (ribo)nucleases and/or RNAses may be provided or added, rather than just relying on enzymes inside the cells.

[0056] The preferred RNA reduction technique therefore involves the transfer of RNA from inside the fungal cell to the outside of the fungal cell, for example into a surrounding aqueous liquid (e.g. the broth). The cells can later be separated or removed from this aqueous liquid

[0057] If heat treatment is employed, the fungal cells can be heated to a temperature of from 55 to 150°C, such as from 80 to 100°C, optimally from 90 to 95°C. This may be for a time from 5 to 120 minutes, such as from 20 to 60 minutes, optimally from 25 to 40 minutes.

[0058] One other method of RNA removal is to subject the fungal cells to an acid or alkaline pH. If an acid pH is provided, this may be from 3 to 4.5, such as from 3.5 to 4.2. This acid treatment may last from 15 to 120 minutes, such as 30 to 60 minutes. It may, if necessary, be combined with heat treatment, such as from 75 to 95°C, such as from 80 to 90°C. Suitable acids include inorganic acids such as hydrochloric acid, phosphoric acid, nitric acid and/or sulphuric acid.

[0059] If an alkaline pH is provided, this may be from a pH of 8 to 12, such as from 9 to 11, optimally at a pH of from 8 to 10. The alkali may be provided by ammonia, alkali or alkaline earth metal oxides, hydroxides or carbonates. The alkali treatment may be for the same time as specified for the acid treatment, and may optionally also be accompanied by heat treatment as described for the acid treatment. However in some cases a lower elevated temperature may be more appropriate, for example from 50 to 80°C, such as from 60 to 70°C, optimally from 62 to 68°C.

[0060] The fungal cells may be subjected to both acid and alkali treatment, either of which may be combined with heat treatment. Preferably though the RNA reduction is a one stage process. The acid or alkali solution used to contact the fungal cells may be discarded, reused or recycled.

[0061] If necessary the RNA removal may consist of or include a heat shock treatment. This may involve particularly high temperatures, such as 100 to 150°C, optimally 130 to 140°C. This may only last for 30 to 200 seconds, such as from 80 to 120 seconds. This heat shock treatment may be provided after acid and/or alkali treatment.

[0062] Whichever RNA content reduction technique(s) are employed, it is desirable that the fungal cells remain intact, or whole, in other words are not lysed. The cells should thus be intact but not alive (e.g. non-viable).

[0063] The fungal cells after RNA reduction preferably have an RNA content below 4.0% or 2.0%, such as from 0.1 to 2.0%, preferably from 0.5 to 1.5%. Optimally the RNA content is from 0.4 to 0.8%. These percentages are based on the dry weight of the cells. Cells with a reduced RNA content may thus have a content below that of the naturally occurring fungus or the fungus used in the fermentation process (stage (a) of the first aspect).

[0064] The fungal cells may then be subjected to heat treatment (either as provided for RNA reduction, or in addition) and/or pasteurization. Alternatively this stage could be thought of as sterilization or the inactivation of undesirable proteins or enzymes, for example proteases, lipases, amylase, phospholipases and/or lipoxygenases. This step (as with RNA reduction) may be performed either if the fungal cells are still in an aqueous liquid (for example the broth, e.g. while still in the fermented vessel) or (preferably) if they have been subjected to one or more water removal steps. Here the heat treatment may either reduce water content and/or make the fungal cells more water-insoluble.

[0065] The protein produced may be located in various parts of the fungal cell. It may represent up to 30%, such as up to 40% and optimally up to 50% of the fungal cell itself (based on dry weight). The fungal protein may be inside the cell (intracellular) or inside the cell wall. The former may include two different "types" of proteins, for example structural proteins (those concerned with DNA; ribosomes; membranes etc) and catalytic proteins (for example enzymes). Cell wall proteins include not only those that are inside or part of the cell wall, but may be outside of the cell wall but still bound to the cell wall. This is contrast to secreted proteins that are not bound to the cell, and which are usually discarded or otherwise lost during processing. The material containing the fungal cells may then be subjected, if necessary, to a (further) water removal step, or de-watering. This will preferably reduce the water content to from 50 to 90%, such as from 55 to 80%, optimally from 60 to 75%. The liquid removed at this stage preferably contains the RNA, RNA degradation products or any other undesired substances either removed from the cells or transferred from the inside the outside of the cells, in the previous RNA reduction or heating stage(s). Procedures for removing the water here are the same as described for the optional removal step earlier following fermentation. However, at this stage filtration is preferred, such as vacuum filtration.

[0066] At this stage in the process one can have produced the proteinaceous substance that is the subject of the third aspect of the invention. It may be in the form of an (e.g. aqueous) paste. Further processing, in particular texturising, for example using mechanical methods, can then be performed in order to produce the edible (proteinaceous) textured product of the fourth aspect. Other processing techniques may include chemical, physical and/or enzymatic treatment.

[0067] To the fungal cells (of the second aspect) one may add or mix in one or more edible components. These may be to add texture and/or flavour. Preferred components include hydrocolloids, for example pectin, starch, carrageenan or alginate.

[0068] Also contemplated are proteins, for example milk protein such as casein, egg albumin, vegetable proteins such as soy, or cereal proteins, such as gluten, or enzymes (e.g. proteases, phosphodiesterases).

[0069] Other edible components include flavour enhancers such as salt, sugar, IMP and/or GMP (although in this case it will be preferred that the RNA level does not exceed those mentioned earlier for the fungal cells), flavouring agents such as spices, herbs, proteins (e.g. from 2 to 5% such as a milk protein, e.g. casein, a vegetable protein, an ovoprotein, e.g. albumin), hydrocolloids (e.g. from 5 to 20% such as pectin, carageenan, agar, xanthan, gellan, galacturonic or mannuronic acid or salts thereof), flour, alginate (such as 0.2 to 1.0%), edible polymers (e.g. cellulose, methylcellulose) , gelling agents (such as egg albumin, whey protein and alginate), polysaccharides (such as from 0 to 10%, for example starch or pectin), colouring agents, plant material such as vegetables (onions, carrots, soy, peas, beans or cereals such as wheat, oats, barley) and emulsifiers. It may also include meat-like flavourings, such as beef, pork or poultry (chicken or turkey) flavourings or other non-meat products. Additional components may be provided to improve taste (organoleptic properties) to improve water binding, fat binding, emulsification properties, texture, volume, viscosity, flavour, aroma and/or colour (dyes, carotenoids, etc.). Egg albumin may be included to improve whippability, colouring or as a binder of other proteins. Egg yolk can be used for emulsification, colour or flavour. Soy protein can be employed for water binding, fat binding and to improve texture. Gelatin can be included to improve gelation. Milk protein or salts thereof for water binding and fat binding flavour or texture and wheat gluten for water binding, texture or flavour. The edible proteinaceous product may therefore be used to replace or be provided in addition to one or more of the following: vegetable proteins, egg white, gelatin, edible proteinaceous foaming agents and milk proteins.

[0070] The texturization is intended to provide texture to the product so that it has meat-like texture and/or it has a mouthfeel similar to meat. It may thus have a fibrous or meat-like appearance.

[0071] Texturization is preferably by one or more mechanical means. These include milling, crumbling, mincing, slicing, cutting (e.g. into chunks, slices or layers), kneading, layering, rolling, sheeting and/or extruding. Preferably it may result in the alignment of the fungal protein into fibres, which may assist to give the product the appearance of meat.

[0072] Here the product may consist of a matrix of hyphae of the fungal cells, where the hyphae entwines and binds the cells together. Preferably the product may then have fibre bundles.

[0073] The texturising may however comprise physical methods, for example heating and/or freezing. Both of these techniques may also result in further water removal. Freezing in particular may assist in alignment of the fungal cells into a fibrous appearance.

[0074] The mechanical shaping may include placing the fungal cells and fungal protein into a mould or other container of a desired shape, and then cooling (such as freezing) and/or (heating, for example 70 to 100°C (to cause gelling, for example). Pressure may be applied if necessary. The combination of cells and protein can then be removed from the mould or container, and can retain the shape of that container.

[0075] The shaped product may for example be in the shape of animals, birds or fish, letters of the alphabet, numbers, etc which may be particularly suitable for foodstuffs for children.

[0076] The proteinaceous product may therefore be in a variety of forms. For example, it may be in the form of chunks, for example meat-like chunks, sheets, granules, slices or may be layered. These forms may be dried or frozen. These chunks may be included into the foodstuff as they are or with little additional processing. They will therefore be recognizable as chunks in the foodstuff, and may have the appearance of meat.

[0077] They can thus be included in foodstuffs as meat substitutes, such as in ready-made meals, pies, microwaveable meals, savoury snacks.

[0078] The textured product may be in the form of pellets or granules, and these too may be dried or frozen. They may be adapted for dehydration before consumption. These products may be included in soups or sauces. However when combined together, for example with an additional (e.g. edible) binder, the pellets or granules may be included in burgers or sausages. A suitable sausage preparation process and sausage making machine is described in the European patent application entitled "Foodstuff with skin containing protein and hydrocolloid" filed in September 1998 in the name of Gist-brocades B.V.

[0079] The textured product may also be in the form of a dried powder, which may be included in a foodstuff to increase mouthfeel or to increase viscosity.

[0080] A particularly preferred texturization method involves granulation, for example to produce granular particles. Before any texturization, the combined fungal cells and fungal protein may have an average water content of from 20 to 75%. After texturing (e.g. granulation), the resulting granules may have an average water content of below 30%, e. g. less than 20%, optionally less than 10%.

[0081] Preferably granulation is achieved using extrusion. This is preferred because extrusion conditions can be adjusted to minimise disruption of the fungal cells. Extrusion may therefore be conducted without heating, for example at from 20 to 30°C. During extrusion the granules may form naturally, falling away under their own weight (from the die plate, such as by gravity) or one can use a cutter, such as a rotating blade, to cut the long strands of "spaghetti" produced by the extrusion. Following extrusion the granules preferably have a water content less than 15%, such as less than 10%, and optimally from 3 to 7%. The granules may have a diameter of from 0.3 to 10mm, such as from 0.7

to 5mm, optimally from 1 to 3mm.

[0082] Extrusion may thus be used to form elongate "spaghetti" like products (these may be cylindrical and/or of circular cross-section) if passed through a suitable die-plate (e,g, with circular or square holes). However formation into for example sheets or layers can be achieved by passage (e.g. using extrusion) through one or more slots. These forms may also be prepared by the use of one or more moving surfaces, such as roller(s) and/or cylinder(s). These may be moving in the same direction or counter-rotating and there may be one, two or up to five such surfaces.

[0083] A particularly preferred process of the present invention may therefore comprise:

1. fermenting fungal cells of the order *Mucorales,* for example in an aqueous liquid contained in a fermenter vessel, the liquid comprising assimilable nitrogen and carbon sources. The liquid and cells can be mixed during fermentation and if necessary depelleting can be performed;
2. optionally removing water, for example from the aqueous liquid, preferably using mechanical techniques such as filtration, centrifugation, settling and/or drying;
3. reducing the RNA content of fungal cells, for example by physical, chemical and/or enzymatic treatment(s);
4. heat treating or pasteurizing the cells or otherwise inactivating undesirable proteins or enzymes inside the fungal cells;
5. optionally, removing water, such as to provide a proteinaceous substance of the third aspect;
6. adding to the fungal cells one or more edible components;
7. texturising the fungal cells (either before or after edible component addition in stage 6), for example using mechanical processing;
8. subjecting the fungal cells to physical treatments such as heating and/or freezing, or otherwise removing water;
9. shaping and/or otherwise mechanically processing the resulting product; and
10. including the edible proteinaceous product into a foodstuff.

[0084] A fifth aspect of the present invention relates to a foodstuff which comprises a textured edible product either of the fourth aspect or preparable by a process of the second aspect. As will be expected, the foodstuff may contain one or more additional edible components or ingredients in addition to the fungal cells. These may be the same as those described above in relation to the proteinaceous product.

[0085] The textured product can be included into the foodstuff as it is, in other words it may simply be used to supplement an existing foodstuff or it may be used in the preparation of a foodstuff. It may be heated first to generate nicer flavours or to brown the product.

[0086] A sixth aspect of the present invention therefore relates to a process for the preparation of a foodstuff, a process comprising either supplementing a foodstuff with the edible textured product of the third aspect, or adding the textured product to one or more components or ingredients of a foodstuff.

[0087] Preferred foodstuffs include ready-made or convenience meals, or microwavable meals, burgers, pies, pasties, sausages and soups. The product can be used a substitute for meats such as pork, beef, poultry, game, ham, veal or even fish.

[0088] These foodstuffs are of course intended for human consumption, although foodstuffs for animals, in particular pets (such as dogs and cats), such as canned foodstuffs, are contemplated.

[0089] Other foods can be included as components or ingredients, for example rice and pasta.

[0090] Preferred features and characteristics of one aspect of the invention are applicable for another aspect *mutatis mutandis.*

[0091] The invention will now be described by way of example with reference to the accompanying Examples, which are provided merely for the purposes of illustration and are not to be construed as being limiting.

EXAMPLES

Comparative Example 1: Selection of suitable microorganisms

[0092] The microorganism needs to be food grade and the substance should contain "valuable" proteins. The essential nutrients as in meat should also preferably be present. The morphology/structure of the biomass has to be suitable to produce a mycoprotein enriched product with a "bite" and organoleptic sensation of meat-like products.

[0093] The Examples demonstrate that manufacturing fungal food from *Mucorales* fungi is feasible, and that the more "primitive" families within the *Mucorales* order can be preferable.

[0094] Advantages of *Mucorales* fungi include:

1. Low or absent mycotoxin production;
2. Simple and cheap biomass production: good growth to high concentrations in clear media (composed of salts,

a well-defined complex N-source, and glucose or oligosaccharides);
3. Down-stream processing procedures are acceptable for foodstuffs; and
4. Good quality of end product.

Flask experiments

[0095] In flask experiments strains were tested belonging to the *Mucorales* families of *Choanephoraceae, Mucoraceae,* and *Mortierellaceae* to test their performance with regard to the following criteria:

- hyphal growth; and
- growth in simple and clear media.

[0096] Growth was tested several times in the following medium, and was always found to be positive:

compound	concentration
yeast extract	5 g/kg
glucose	30 g/kg
potassium phosphate	0.10 M
ammonium sulphate	0.1 M
magnesium sulphate	1.25 mM
zinc sulphate	0.03 mM
manganese sulphate	0.2 mM
iron chloride	0.09 mM
copper sulphate	0.03 mM

[0097] The components were dissolved in deionized water, and sterilized for 20 minutes at 120°C: the glucose was sterilized separately. The initial pH was 6.0.
[0098] The experiments were conducted in Erlenmeyer flasks (100/500ml), and inoculated with a suspension of spores prepared freshly by growing the strains for several days on a malt agar surface, and rinsing the spores from the surface. The spore concentration in the flasks at start was 10^5 - 10^6/ml. The flasks were incubated between 25 and 35°C for 20 to 50 hours on an orbital shaker (with a 2.5 cm stroke at 250 rpm).
[0099] The following strains were tested:

species	family	source
Choanephora infundibulifera	*Choanephoraceae*	CBS 155.58
Blakeslea trispora	*Choanephoraceae*	CBS 131.59
Gilbertella persicaria	*Choanephoraceae*	CBS 247. 59
Absidia pseudocylindrospora	*Mucoraceae*	CBS 100.2
Phycomyces blakesleeanus	*Mucoraceae*	CBS 226.92
Rhizopus oryzae	*Mucoraceae*	own isolate
Mucor hiemalis	*Mucoraceae*	CBS 242.35
Rhizomucor miehei	*Mucoraceae*	own isolate
Morierella alpina	*Morierellaceae*	CBS 528.72; CBS 168.95

[0100] For many strains specific culture conditions were required to force them into a dispersed mode, otherwise they will grow in the pellet form. Measures were taken when appropriate to obtain the dispersed mod and reduce the number of pellets (by sonication or high shear mixing).
[0101] For some strains it was necessary to grow the spores first in a more complex medium (soybean meal, 20 g/kg and glucose, 30 g/kg) to obtain dispersed mycelium that then was transferred to the test medium.
[0102] Mixing conditions were adapted for several strains: if necessary flasks with a baffle were used, or the filling level of the flask and/or agitation rate was adjusted.
[0103] In all cases, however, it was possible to obtain 2 g/kg biomass in the course of incubation, measured by filtering the biomass and weighing it after drying for 24 hours at 105°C on a preweighed filter paper. The presence of pellets was checked visually. In most strains they appeared to be present but only in minor quantities. Microscopic

inspection revealed that the morphology always was hyphal based.

Comparative Example 2: Fermentor experiments

[0104] As part of the scale-up process all the strains were subjected to lab scale fermentor experiments. The objective was to test them using the following criteria:

- hyphal growth;
- growth in simple media; and
- growth to high biomass concentration in a process that allows further scale-ups.

[0105] The experimental set up was as follows, starting with inoculum preparation.
[0106] The spore suspension was prepared as described in the previous Example. With this spore suspension an inoculum culture was started, using the media described in Example 1 (either the clear medium with yeast extract, or the soy bean containing medium). As soon as full growth had been reached the culture was transferred to a lab fermentor, containing medium that was prepared using the following components:

component	concentration (g/kg)
yeast extract	19
glucose	45
ammonium sulphate	13.2
magnesium sulphate.7 H_2O	2
calcium chloride	0.5
potassium monophosphate	3
zinc sulphate.7H_2O	0.0144
iron sulphate.7H_2O	0.15
manganese sulphate.1H_2O	0.0228
copper sulphate.5H_2O	0.0024
cobalt sulphate.7H_2O	0.0038
thiamine.HCl	0.004
nicotinic acid	0.002

[0107] All compounds were dissolved in deionized water and mixed, except the glucose which was prepared separately. The pH was adjusted to 6.0 using NaOH, and the medium was sterilized in the fermentor for 45 minutes at 121°C in an autoclave. The glucose solution was added after separate sterilization for 20 minutes at 120°C, after acidification to pH 5 with phosphoric acid.
[0108] Next to the batch medium a carbohydrate feed was supplied which consisted of glucose at a concentration of ca. 500 g/kg. The preparation was as described for the glucose solution of the batch medium.
[0109] The fermentor was equipped with temperature, pH and foam control. To adjust the pH solutions of ammonia and sulphuric acid were used. Moreover dissolved oxygen concentration and the composition of the liberated gas was measured. The culture was aerated, using ca. 1 volume of air per volume of broth per minute. Mixing was intensive using Rushton turbines and baffles. The glucose feed was applied at a rate between 1 and 5 g/glucose/kg broth/hour. It was started when the glucose concentration in the broth had decreased to a concentration below 5 g/kg.
[0110] Samples were taken twice every 24 hours for off-line analysis of concentrations of unused substrate, biomass and by-products. Moreover, microscopic inspection was performed.
[0111] A selection was made of the following strains tested in flasks for experiments in lab scale fermentors:
Rhizopus oryzae, Rhizomucor miehei, Mortierella alpina and *Blakeslea trispora.*
[0112] In all cases the morphology appeared to be hyphal as observed under the microscope. Pellets were observed visually, but not in significant numbers. Biomass accumulated to concentrations beyond 40 g/kg within 80 hours of cultivation.
[0113] All strains thus indicated potential to be able to produce biomass at low cost, better so when further scaling up the process.

Comparative Example 3

[0114] In shake flasks various microorganisms were cultivated according to the procedures given in Example 1. The

morphology of the biomass was examined by light microscopic methods. The characteristics found are shown in Table 1.

TABLE 1

Strain	Identification length (μm)	Diameter (μm)	Branched
Fusarium graminareum	300 - 500	5 - 8	no
Mortierella alpina	200 - 300	8- 12	yes
Rhizomucor miehei	100 - 150	8 - 10	yes
Rhizopus oryzae	100 - 200	10	yes
Blakeslea trispora	80 - 100	2 - 4	yes

Comparative Example 4

[0115] In lab scale fermentors the various microorganisms were cultivated according to the procedures described under Example 2.

[0116] The dried biomass, harvested at end of fermentation was analysed for the following components: protein, RNA, carbohydrate, fat, ash. The results are shown in Table 2.

TABLE 2

	% of dry matter content			
species	RNA	protein	fat	carbohydrate
Mortierella alpina	8	35	30	15
Rhizomucor miehei	8	45	20	20
Rhizopus oryzae	8	40	25	25
Blakeslea trispora	8	35	20	20

Example 5: Fermentation and production of biomass

[0117] Biomass from strains *Rhizopus, Rhizomucor,* and *Mortierella* obtained in Example 4 was isolated by removing the extracellular broth components by centrifugation in a lab tube centrifuge. Here, the harvested biomass was centrifuged for 15 minutes in a Beckman centrifuge (type J-6M/E) at 15°C. The supernatant was discarded and the pellet was resuspended in a 0.1 M K_2HPO_4 buffer at pH 7. The mixture was recentrifuged.

[0118] The broth was treated for 15, 30 and 60 minutes at 65°C and aliquots of broth and biomass analysed; the results are shown in Table 3 (the percentages are based on dry weight). The analyses as described in Example 3 were performed.

TABLE 3

Fermentation strain	dry matter content (g/kg)	RNA content (%)	Carbohydrate (%)	Lipid content (%)	Protein content (%)
Rhizomucor miehei	220	2.3	22	21	48
Rhizopus oryzae	338	1.8	28	26	42
Mortierella alpina	376	1.7	16	33	37

[0119] After the heat treatment a microscopic examination was performed . The hyphae of both the *Mortierella* and *Rhizopus* strains were intact, those of the *Rhizomucor* were partly intact.

Example 6: Pasteurisation/Enzyme inactivation

[0120] Broth from Example 5 was heat treated as follows:

60 minutes at 90°C, cooled to 25°C and stored for 48 hours at 25°C.

[0121] Aliquots of the treated broth were then analysed, the results are shown in Table 4 (percentages are based on dry weight).

TABLE 4

Fermentation strain	dry matter content (g/kg)	RNA content (%)	Carbohydrate (%)	Lipid content (%)	Protein content (%)
A. *Rhizomucor miehei*	. 224	2.1	23	21	47
B. *Rhizopus oryzae*	347	1.7	27	26	32
C. *Mortierella alpina*	383	1.7	16	34	36

Example 7: Scaled-up production

[0122] Fermentations of the *Rhizopus oryzae* strain used in Example 5 were performed in a large scale fermenter with a working volume of $3m^3$. The fermentation conditions were scaled up from the conditions as described in Example 4. After fermentation the broth was cooled to 5-10°C and harvested.

[0123] Analyses were performed as described in Example 6, as well as a microscopic examination.

Example 8: Preparation of edible biomass

[0124] The broth obtained from Example 7 was centrifuged in a Westfalia NA7 disc separator. The centrifuge was provided with 4 nozzles each of a diameter of 1mm. Two streams of fluid were obtained. The supernatant ($3m^3$) was discarded and a concentrate stream that contained the biomass (fungal cells) retained. The concentrate was diluted to the original volume with a 100mM solution of K_2HPO_4.

[0125] The mixture was centrifuged as described for Example 5 and supernatant was discarded.

[0126] The washed concentrated biomass was then heated to 65°C for 30 minutes. A sample was taken and immediately cooled down to 5-10°C. The main stream was further heated to 90-95°C and kept at that temperature for 30 minutes.

[0127] The resulting mixture was filtered in a membrane filter press with a filtration area of $6m^2$ and a pressure of 0.3-2 bar. The obtained filter cake was washed with cold mains water (5-10°C). The filter cake was squeezed with the membranes at a pressure of 6 bar.

[0128] This resulted in 250 kg filter cake with a dry matter of 45%.

Examples 9A and 9B: Sheeting, layering, rolling

[0129] The filter cake from Example 8 was milled and crumbled in portions of approximately 100 kg by a Lödige high shear mixer for 5 minutes. To the crumbled cake a mixture of 4kg egg albumin (Example 9A) was added and the mixture kneaded.

[0130] The mixture was formed into sheets of 1mm by rolling equipment.

[0131] The sheets were heated to 80°C in an ventilated oven or tunnel. The sheets were layered and rolled in the form of a "Swiss roll". The roll was frozen to -20°C using liquid carbon dioxide.

[0132] The same procedure was repeated except 4kg pectin (Example 9B) was substituted for the egg albumin.

Example 10: Food additives

[0133] The frozen biomass as from Examples 9A and 9B was thawed.

[0134] To the biomass colouring additives, taste enhancing products (spices, vegetables and onions) were added. The mixture was homogenised in a kneader. The homogenised mixture was formed into burgers, packed, pasteurised and frozen.

Example 11

[0135] The frozen biomass from each of Examples 9A and B was thawed, and to the biomass colour additives,

spices, vegetables (onions) were added. The mixture was homogenised in a kneader. The homogenised mixture was extruded into a continuous tube (for sausages) with co-extrusion of a (vegetarian) skin-forming material using a continuous sausage-making system to make sausages.

Examples 12A and B

[0136] The filter cake from Example 8 was milled and crumbled into portions of approximately 100kg by a Lödige high shear mixer for 5 minutes. To the crumbled cake 4kg egg albumin was added and the mixture kneaded. The kneaded mixture was extruded with a single screw extruder with a dieplate with holes of 1mm. The extrudate was transported by a belt and dried in a fluidised bed drier (air temperatures of 50°C) to form granules. For Example 12B pectin was used at the same amount instead of egg albumin.

Example 13

[0137] A batch of 25kg of the dried extrudate from each of Examples 12A and B was mixed with 60kg mains water. To the mixture the food additives in Example 10 were added and the mixture kneaded and formed into burgers.

Example 14

[0138] A batch of 25kg of the dried extrudate from each of Examples 12A and 12B was mixed with 60kg mains water. To the mixture the food additives from Example 11 were added and the mixture processed into sausages as described in Example 11.

Example 15

[0139] 25kg of the frozen biomass from each of Examples 9A and B was thawed and to the biomass colour additives and taste enhancing products (spices, vegetables, onions) were added. To the mixture 1kg vegetable fibres (cellulose fibres with an average fibre length of 300-1000μm) was added and homogenised in a kneader. The homogenised mixture was formed into burgers, packed, pasteurised and frozen.

Example 16

[0140] Frozen biomass (25kg) from each of Examples 9A and B was thawed and to the biomass colour additives, spices, vegetables and onions were added. To the resulting mixture 1kg vegetable fibres (cellulose fibres with an average fibre length of 300-1000μm) was added and the mixture homogenised in a kneader. The homogenised mixture was formed by extrusion into sausages by co-extrusion with a vegetarian skin as described in Example 11.

Example 17

[0141] The filter cake from Example 8 was milled and crumbled in portions of approximately 100kg by a Lödige high shear mixer for 5 minutes. To the crumbled cake a mixture of 1kg egg albumin was added and 4kg vegetable fibres (cellulose fibres with an average fibre length of 300-1000μm). The mixture was kneaded and then extruded in a single screw extruder with a dieplate with holes of 1mm. The extrudate was transported by a belt and dried in a fluidised bed dryer (air temperature of 65 to 80°C), to form granules. These were then added to a soup and to dried soup powders (that form soups on rehydration).

Example 18

[0142] A batch of 25kg of the dried extrudate from Example 17 was mixed with 60kg mains water. To the mixture the food additives as described in Example 15 were added and the mixture kneaded and used to make burgers as described in Example 15.

Example 19

[0143] A batch of 25kg of the dried extrudate from Example 17 was mixed with 60kg mains water. To the mixture the ingredients as described in Example 16 were added, the mixture kneaded and used to form sausages as described in Example 16.

Claims

1. A process for the preparation of a proteinaceous substance, suitable for use in a foodstuff, comprising fungal cells, the process comprising:

    a. fermenting fungal cells of the order *Mucorales* in an aqueous liquid contained in a fermenter vessel, the liquid comprising an assimilable nitrogen (N) source and an assimilable carbon (C) source which allows the fungal cells to produce protein, and mixing the liquid and cells during fermentation;
    b. reducing the RNA content of the fungal cells;
    c. before or after (b), removing at least some of the water from the mixture of fungal cells; and
    d. processing the fungal cells into an edible substance.

2. A process according to claim 1 wherein the liquid and fermenter are devoid of any food and/or the fungal cells constitute at least 50% of the proteinaceous substance.

3. A process for the preparation of an edible textured product, the process comprising mechanically texturising an edible proteinaceous substance comprising fungal cells of the order *Mucorales* with a reduced RNA content.

4. A process according to claim 3 wherein the proteinaceous substance is preparable by a process according to claim 1 or 2.

5. An edible proteinaceous substance suitable for use in a foodstuff comprising fungal cells of the order *Mucorales* having a reduced RNA content.

6. A substance according to claim 3 to 4 which is predominantly fungal cells and/or the fungal cells constitute the only edible components.

7. A substance according to claim 5 or 6 wherein the fungal cells constitute at least 70% of the substance.

8. An edible textured product comprising fungal cells of the order *Mucorales* having a reduced RNA content.

9. A foodstuff which comprises a textured product either as defined in claim 8 or produceable by a process according to claim 3 or 4.

10. A foodstuff according to claim 9 comprising one or more edible component(s) in addition to the fungal cells.

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EUROPEAN SEARCH REPORT

Application Number
EP 98 30 7450

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	22 January 1999	De Jong, E

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EUROPEAN SEARCH REPORT

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